Quenching of Fluorescent Nucleotides Bound to Myosin: A Probe of the Active-Site Conformation†

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ABSTRACT: The conformation of the active ATPase site of myosin subfragment 1 (S1) and actomyosin in myofibrils was probed by measuring the solvent accessibility of the bound ethenonucleotides ϵADP and ϵATP (during steady-state hydrolysis). Solvent accessibility was determined by measuring the quenching of fluorescence produced by the solvent-phase quencher acrylamide, 25–400 mM. The fraction of the nucleotides that were specifically bound to the active site was determined following sedimentation in the presence and absence of 5 mM ADP. In agreement with previous investigations, both ϵATP and ϵADP were almost completely protected from the quencher when bound to the active site of myosin. The solvent accessibility of both ϵADP and ϵATP varied with both temperature and ionic strength. The nucleotides became more accessible at higher temperatures and higher ionic strength. At 1 M KCl the quenching curve was biphasic, indicating that the nucleotide pocket of myosin can exist in both a closed form that allows little quenching and a more open form that allows considerable quenching. However, the transition between forms was not strongly coupled to the state of the nucleotide, with a similar protection observed for both ϵADP and for ϵATP during steady-state cycling. ϵADP bound to acto-S1 or to actomyosin in myofibrils displayed the same degree of protection as seen with S1 alone. A similar result is obtained during steady-state hydrolysis. Thus nucleotides in the myosin pocket do not become more accessible to the solvent when myosin binds to actin in either rigor–ADP or active complexes. These results support the hypothesis that the nucleotide pocket can exist in both open and closed forms, but they suggest that transitions between these forms are not involved in the powerstroke.

The structures of both actin and the myosin head have now been determined to a resolution of 2.8 Å (Kabsch et al., 1990; Holmes et al., 1990; Schutt et al., 1993; Rayment et al., 1993a). The structure of S1 is composed of a large globular region connected to a more slender neck. The globular region, known as the catalytic domain, contains sites for binding both actin and nucleotide. The long tapering neck extends from this globular region to the junction with the rod, which leads in turn to the thick filament. The catalytic domain contains a region which has homology to ATP binding sequences from other proteins. This portion of the structure consists of a shallow depression in the protein structure termed the nucleotide pocket. The pocket is large and open, and two amino acids identified by cross-linking to photoreactive nucleotides reside on opposite sides, spaced 15 Å apart (Yount et al., 1992). The conformations of a number of enzyme nucleotide binding sites are now known, and most of these exist in a more closed form in which the protein structure encloses the nucleotide, isolating it from the solvent (Schultz, 1991). The exclusion of solvent from the active site prior to nucleotide hydrolysis is thought to be necessary in order to exclude water from the reaction. For instance, adenylate kinase, an enzyme whose active site displays some homology with that of myosin, undergoes a domain shift that encloses the bound nucleotides prior to the phosphate transfer (Schultz et al., 1991). These structures suggest that, at some point in the cycle, myosin may undergo a domain shift which closes the nucleotide pocket. As outlined below, such domain movement could play a crucial role in the generation of force.

It has been suggested that opening and closing of the nucleotide pocket accompanies the protein conformational changes that drive the powerstroke (Rayment et al., 1993b). The atomic structures of actin and myosin have been "docked" into macromolecular complexes, to produce atomic models of the actin filament and of the actin filament complexed with the myosin head (Schroder et al., 1993; Rayment et al., 1993b). The structure of the acto-S1 complex led Rayment et al. (1993b) to suggest a hypothesis for how motion is produced during the interaction of these two proteins. In the structure of the actomyosin complex, the opening of a previously closed nucleotide pocket could swing the neck region of the myosin head through a considerable angle, resulting in a movement of the head–rod junction by approximately 5 nm or more in the direction required for producing mechanical work. The production of useful work, i.e., the powerstroke, is associated with the release of products occurring after myosin has bound to actin [for reviews see Cooke (1986) and Goldman (1987)]. These two observations suggest the following sequence of events. The binding of ATP or of the hydrolysis product, ADP·P_i, to myosin causes the nucleotide pocket to close. The myosin with a closed pocket binds to actin, with the catalytic region rigidly oriented on the actin filament. After binding, the release of products results in the opening of the nucleotide pocket. Opening of the pocket results in a shift in the orientation of the neck, generating a powerstroke of some 5–7 nm. Central to this hypothesis is the cyclical opening and shutting of the nucleotide pocket. In particular, the low-energy state of the pocket in the absence of nucleotides would be the open configuration. The closed configuration could only be achieved by the tight binding of ATP, which provides some 50 kJ/mol free energy. The binding of the head to actin with the subsequent release of products would result in an

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open pocket at the end of the powerstroke. Because the binding of ADP to actomyosin or to rigor fibers causes little change in the orientation of the myosin or in the mechanics of the fibers, the actomyosin·ADP state should be a good representation of the end of the powerstroke with an open pocket (Fajer et al., 1990; Dantzig et al., 1991). To test these hypotheses we bound fluorescent nucleotides to the nucleotide pocket to monitor changes in its conformation during force generation.

Two fluorescent nucleotides, εADP and aza-εADP, have been used extensively for studying the conformation of the myosin site pocket. These both involve modifications of the adenine ring, producing a fluorescent base. It has been shown that these nucleotides bind to the myosin head and that they are substrates for hydrolysis. However, the rates of a number of the transitions in the cycle are perturbed by the presence of the etheno ring (Garland & Cheung, 1976; Rosenfeld & Taylor, 1984; Smith & White, 1985). Both nucleotides support full relaxation of skinned rabbit fibers and support active tensions and velocities that are approximately one-third that of ATP (Pate et al., 1992). Ando, Morales, and co-workers first showed that εADP and εATP are less available to solvent-phase quenchers when they are bound to the nucleotide site in isolated myosin (Ando et al., 1992). Rosenfeld and Taylor (1984) found that εADP bound to myosin existed in two forms, one of which was less accessible to acrylamide quenching than the other. They further found that during steady-state hydrolysis the nucleotides were protected to the same extent as was εADP and that in the complex myosin·εADP·V_i the accessibility of the nucleotide was still further reduced. These data suggest that the nucleotide pocket does indeed close around the nucleotide as suggested by Rayment et al. (1993b); however, they do not define the energetics of the conformational change that occurs in this process. To explore the energetics of the closing of the pocket we have measured the solvent accessibility as a function of temperature and solvent conditions. We find that as temperature or ionic strength is increased, bound nucleotides become more accessible to the solvent, suggesting that the pocket opens, but that this process is not strongly coupled to the state of the bound nucleotide. A second crucial aspect of the theory proposed by Rayment and co-workers, discussed above, is that the nucleotide pocket should open after the myosin binds strongly to actin. To test this hypothesis we measured the solvent accessibility of εADP bound to acto-S1 and to actomyosin in myofibrils. We found that the nucleotides remained protected by the protein structure in the actomyosin·εADP complex and that they remain protected even during steady-state hydrolysis. These results question whether the opening of the myosin nucleotide pocket is tightly coupled to the production of work in the powerstroke.

MATERIALS AND METHODS

Protein and Nucleotide Preparations. Myosin was prepared from rabbit back and leg muscles by the method of Tonomura et al. (1966). S1 was prepared from this myosin using digestion by α-chymotrypsin following the method of Weeds and Taylor (1975). Actin was prepared from an acetone-dried muscle powder by the method of Spudich and Watt (1971). Myofibrils were prepared using a modification of the method of Etlinger et al. (1976). The modification involved inclusion of the protease inhibitors phenylmethanesulfonyl fluoride (PMSF), 0.1 mM; leupeptin, 0.01 mM; and aprotinin 100 units/mL, in the initial extraction and sedimentation. PMSF was also included in all subsequent steps. Myofibrils were stored at −20 °C in a solution containing 50% glycerol and 50% rigor solution (described below). Protein was determined by the method of Bradford (1976). The molecular weight of S1 was taken as 120 kDa, and the content of myosin in myofibrils was taken to be 45% of total protein (Cooke & Franks, 1980). εADP was obtained from Molecular Probes (Eugene, OR). Thin-layer chromatography on cellulose polyethylenimine plates showed that the material was >95% pure. Crystalline acrylamide was obtained from Bio-Rad. Spectra were obtained in a low ionic strength rigor solution consisting of 20 mM MOPS, 5 mM $MgCl_2$, and 1 mM EGTA, pH 7.0, with additions as noted. Experiments were performed at 20 ± 0.5 °C, except where noted.

Some of the samples consisted of myofibrils loaded with S1. Myofibrils were first incubated in a slight excess of S1, assuming that 1.7 additional unoccupied actin sites existed for each myosin head in the myofibrils. The myofibrils were then sedimented to remove excess unbound S1. The loaded myofibrils were resuspended and an additional aliquot of myofibrils (equal to 10% of the S1-loaded myofibrils) was added to provide an excess of actin sites over S1. This would ensure that virtually all S1 would remain complexed to actin following the addition of εADP.

Fluorescence Measurements. Steady-state fluorescence was measured using an SLM fluorometer (interfaced to an IBM PC-XT), with excitation at 320 nm and emission at 410 nm. Both excitation and emission slits were at 2.5 mm, producing bandwidths of 10 nm. Fluorescence was collected using the front-face optical path from cells that were 1–2 mm thick. The optical density of the most turbid samples, 20 mg/mL myofibrils, was 1.8 at a wavelength of 320 nm and 1.4 at a wavelength of 410 nm. Although the turbidity of the samples affected the absolute value of the fluorescent signal, all data were analyzed in terms of Stern–Volmer plots that involve ratios of fluorescence intensities. Because the turbidity of the samples was unaffected by acrylamide, its effect on the ratio of two intensities is minimal.

The decrease in fluorescence intensity upon addition of acrylamide was due both to quenching of the fluorescence by the acrylamide and to dilution due to the added volume. To minimize the effects of dilution, a concentrated stock solution of acrylamide (0.5 M) was used, producing a total sample dilution of only 7%. This dilution was taken into account in calculating the quenching by the acrylamide. Temperature was maintained by a water-cooled cuvette holder in the fluorometer. The sample was allowed to equilibrate for 10 min prior to obtaining data; measurements of the temperature within the sample using a thermocouple demonstrated that little change, <0.5 °C, occurred during the experiment.

Determination of the Fraction of Bound Nucleotides. An important aspect of the data analysis was the determination of the fraction of nucleotides that were bound to the proteins. This was measured for most of the experimental preparations. For the soluble S1 experiments, the protein nucleotide mixture was sedimented through a semipermeable membrane with a molecular mass cutoff of either 10 or 30 kDa (Centricon). The membrane allowed passage of the free nucleotide but not the protein and bound nucleotide. The sedimentation was stopped when approximately 10–25% of the solution had passed through the membrane and the fluorescence of this solution was measured to determine the concentration of the free nucleotide. In the case of acto-S1 or myofibrils the free nucleotide was determined from the supernatant following sedimentation of the proteins and bound nucleotides in an Airfuge at 100000*g* for 30 min. Sedimentation in the presence of 5 mM ADP or ATP, which displaced the bound nucleotide, allowed determination of the fraction of the fluorescent nucleotide that was bound specifically to active sites.

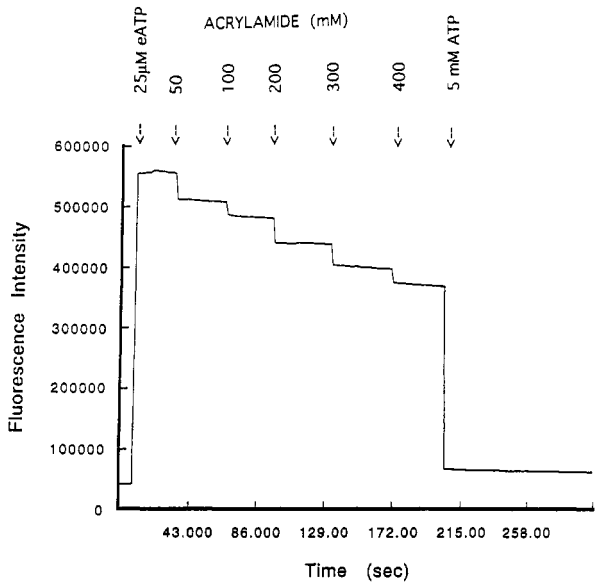

FIGURE 1: Fluorescence is shown as a function of time for 25 μM εADP in the presence of 63 μM S1. Fluorescence was excited at 320 nm and emission was observed at 410 nm. Fluorescence was observed in the front face mode, using cells which have 2-mm path lengths. The initial signal arises from S1 alone. The intensity increases upon addition of 25 μM εATP, which is rapidly hydrolyzed to εADP. Acrylamide was then added to the concentrations shown, producing a decrease in the fluorescence. Data were not collected during the approximately 10 s required for addition of acrylamide, ATP, etc. ATP is added at the end of the run to displace bound εADP from S1, producing a large drop in the intensity due to the quenching of the free nucleotide. The proteins were in low ionic strength rigor solution at 20 °C.

RESULTS

Quenching of Ethenonucleotides Bound to S1. Figure 1 shows a typical experimental protocol for measuring the quenching of the fluorescence of εADP bound to S1. In the presence of S1 alone, the intensity was very low. Addition of 25 μM εADP to the 60 μM S1 resulted in a large increase in the observed intensity. Subsequent additions of aliquots of acrylamide resulted in a decrease in the fluorescence intensity. Following the addition of acrylamide, the addition of ATP caused a large drop in the fluorescence intensity, due to the displacement of bound εADP from the nucleotide pocket. This drop in fluorescence was taken as a measure of the fluorescence of εADP bound specifically to active sites in the sample. Previous studies have shown that the vast majority of these sites are on myosin (Yanagida, 1981). The final resulting fluorescence intensity is approximately that expected for the 25 μM εADP free in a solution containing 400 mM acrylamide. As found by previous workers, εADP bound to myosin is highly protected from quenching by acrylamide. This shows that the nucleotide is effectively enclosed within the protein structure.

The specificity of binding was also investigated by measuring the fluorescence of variable concentrations of εADP added to 25 μM S1 in the presence of 400 mM acrylamide. The fluorescence increased approximately linearly up to 25 μM εADP. The increase in fluorescence above 25 μM εADP was attenuated due to the quenching of the unbound nucleotide. The discontinuity in slope occurred at 23 ± 5 μM added εADP, indicating that binding and protection of the nucleotide occurs stoichiometrically with the active site of S1. In a similar titration of myofibrils, εADP bound and was protected with a stoichiometry of 1.1 ± 0.25 relative to the myosin active sites in the myofibril.

The data shown in Figure 1 can be displayed as Stern–Volmer plots in which the initial fluorescence divided by the observed fluorescence is plotted as a function of the acrylamide concentration. When plotted in this fashion, the data produce a straight line for the case in which a single species is quenched by collision with acrylamide [see Lakowicz (1983) for discussion]. For this case, the fluorescence intensity depends on the acrylamide concentration as follows:

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

where F_0 is the initial fluorescence obtained in the absence of acrylamide, F is the fluorescence obtained in the presence of acrylamide, K_{sv} is the Stern–Volmer quenching constant, and [Q] is the concentration of quencher. The value of K_{sv} is equal to the product of the lifetime of the fluorophore and the effective second-order rate constant for collision between the fluorophore and the quencher. The lifetimes of ethenonucleotides are quenched when bound to S1; however, that of the major component is only decreased slightly from its value in solution, 24 vs 27 ns (Aguirre et al., 1989). Thus to a first approximation, K_{sv} is determined primarily by the accessibility of the fluorophore for the quencher. The quenching of the free nucleotide can be described by this equation, with a value of K_{sv} = 48 M^{-1} shown in Figure 2. When the nucleotide binds to a protein, acrylamide will be prevented from colliding with portions of the fluorescent ring and the slope of the Stern–Volmer plot will be lower with a corresponding decrease in K_{sv}.

As shown in Figure 2, the fluorescence of εADP in the presence of S1 displays a downward concavity characteristic of the quenching of multiple species with different values for the quenching constant K_{sv}. In this case at least two species of nucleotide exist, the free nucleotide and the bound nucleotide. The data were fit using eq 2, which is the appropriate extension of eq 1 to samples which have multiple components (Lakowicz, 1983):

$$F_0/F = 1/(F_1/(1 + K_1]Q]) + F_2/(1 + K_2[Q]) + ...) \quad (2)$$

where F_1, F_2, etc., represent the fluorescent intensities of components 1, 2, etc., in the absence of quenchers and K_1, K_2, etc., represent their respective quenching constants. The data shown in Figure 2 could be fit by a two-component Stern–Volmer equation in which one component represented free nucleotide and the other represented bound nucleotide. The concentration of free nucleotides was first determined as described below.

Measuring the Concentration of Free Nucleotides. Crucial to the interpretation of the quenching of fluorescence is the determination of the fraction of fluorescent nucleotide bound to the myosin active site. The fractions of εADP or εATP bound to S1, to myofibrils, and to acto-S1 were obtained by centrifugation as described in Materials and Methods. These data were then used to determine the fraction F_1 of the free nucleotide in eq 2.

The data listed in Table 1 can also be used to calculate affinity constants K_a. For instance, in the case of S1 listed in Table 1, 80% of the nucleotide was bound, giving 20 μM complex and 5 μM free nucleotide. With a total [S1] of 65 μM and free [S1] of 45 μM, the value of K_a for the binding of εADP to S1 can be shown to be 9×10^4 M^{-1}. The affinity increased slightly as the temperature was decreased. Upon binding of S1 to actin, the value of K_a decreased to 2×10^4 M^{-1} at 20 °C.

The values of the binding constants determined from the data reported in Table 1 are similar to those found by previous workers. The binding of εADP to S1 has been measured by

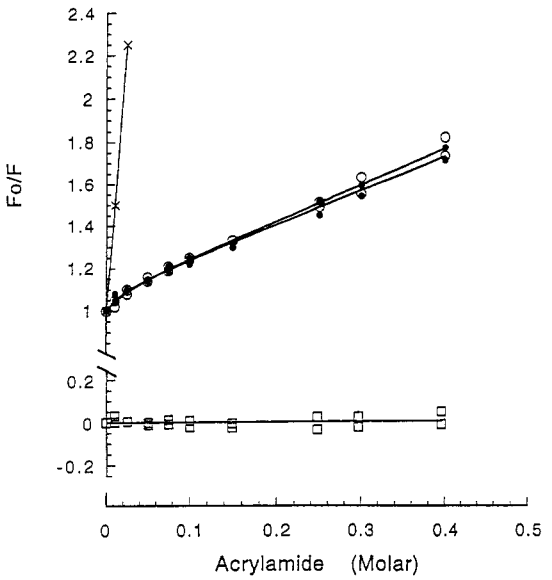

FIGURE 2: F_0/F is plotted as a function of the acrylamide concentration (known as a Stern–Volmer plot). F_0 is the fluorescence observed in the absence of acrylamide, and F is the fluorescence observed in its presence. Data are shown for free ϵADP (×) and for 25 μM ϵADP in the presence of 55 μM S1 in low ionic strength rigor solution in the absence (·) and presence (○) of 10 mM CP and 1 mg/mL CK, producing steady-state cycling of the nucleotide. Data are included for two separate experiments for each condition, to show reproducibility. The temperature was 10 °C. The straight line through the data for free ϵADP defines a Stern–Volmer quenching constant of 48 M^{-1}. The concentration of free ϵADP was determined following sedimentation as described in Materials and Methods. The data were fit using a two-component Stern–Volmer equation, in which one component was free nucleotide as determined by sedimentation and the second component was bound nucleotide, to obtain values of K_{sv}. The values of K_{sv} for bound nucleotide were 1.4 M^{-1} in the presence of S1 and 1.5 M^{-1} in the presence of S1 plus CP/CK, with fractions of bound nucleotides of 0.90 and 0.94, respectively. The data shown below are the residuals of the fit to the data for ϵADP in the presence of S1 (□). They are not statistically different from a straight line. The two-component fit to this data had an R value of 0.998. The data were also fit with a three-component function, protocol 3, described in the text. This fit defined two bound fractions representing 0.44 and 0.46 of the total nucleotide with values of K_{sv} that were 1.45 and 1.41 M^{-1}, respectively. The errors on all of these parameters were huge, indicating that they were cross-correlated. The residual of this fit was 0.998, exactly equal to that obtained with the one-parameter fit. Thus the introduction of a second bound component did not improve the fit over that obtained with one bound component, and the two bound components added together were not different from the single bound component.

several investigators who found slightly tighter binding, with a K_a of (1–2) × 10^5 M^{-1} (Garland & Cheung, 1976; Konrad & Goody, 1982; Rosenfeld & Taylor, 1984). In the presence of 1 M KCl the value of K_a has decreased to 1 × 10^4 M^{-1}, similar to the value obtained by extrapolation of the data of Konrad and Goody (1982), who measured K_a over the range 0.09–0.44 M. The binding of ϵADP to muscle fibers has been investigated by Yanagida (1981). The maximum binding obtained at high ϵADP was 265 μM, close to the concentration of myosin heads within the fiber, indicating that ϵADP bound relatively specifically to the myosin heads. The concentration of the ϵADP which gave half-maximum binding was 355 μM. However, analysis of the data at low concentrations of ϵADP indicated slightly tighter binding with an equilibrium constant of 5 × 10^3 M^{-1} at 25 °C, a little weaker than found here. The binding constant increased as the temperature was lowered to a value of 1.5 × 10^4 M^{-1} at 6 °C. Values similar to that determined above from the data of Table 1 were found for the binding of ϵADP to acto-S1, 1 × 10^4 M^{-1} (White, unpublished), and for the binding of ADP to acto-S1, (1–2) × 10^4 M^{-1} (Biosca et al., 1986).

Table 1: Quench of Ethenonucleotides by Acrylamide

species[a]	% bound[b]	F_b	K_{sv}[c] (M^{-1})
free ϵADP			48 ± 4
S1,	80 ± 5	0.88 ± 0.08	1.6 ± 0.1
S1, CP, CK	85 ± 10	0.84 ± 0.1	1.7 ± 0.15
S1, 1 M KCl	40 ± 10	0.24 ± 0.05	1.2 ± 0.1
		0.15 ± 0.05	16 ± 5
myofibrils	45 ± 5	0.39 ± 0.04	1.1 ± 0.2
myofibrils ± S1	60 ± 10	0.63 ± 0.06	1.3 ± 0.2
active myofibrils	65 ± 10	0.58 ± 0.1	1.5 ± 0.3

[a] All samples were in low ionic strength rigor solution at 20 °C, with the additions noted. The concentration of S1 was 60–70 μM for data listed in rows 2–4, and the concentration of myosin heads in the myofibrils was 45 μM in the last three rows. [b] The fraction of ϵADP bound to the proteins was determined by sedimentation through a semipermeable membrane for samples of S1 or by sedimentation of the proteins for either acto-S1 or myofibrils, as described in Materials and Methods. [c] The value of K_{sv} determined for free ϵADP was used in the fit to the other data. The values for F and K_{sv} of the bound component were then determined by a fit to the data using protocols 2 and 3 as described in the text. Errors are SEM for 5–20 samples. Two components are given for the data in high KCl.

Fitting the Data to the Stern–Volmer Equation. Data were fit to eq 2 using three different protocols. In protocol 1, there were two fluorescent components. Component 1 was taken to represent free nucleotide, with a concentration determined directly from sedimentation and a value of K_{sv} of 48 M^{-1} as determined from the data shown in Figure 2 for the free probe. The bound nucleotide was fit as a single component with a known concentration such that the only independent variable was the value K_{sv} for this component. The data were fit using a nonlinear least-squares algorithm. Data were fit in all cases starting from several different initial guesses to ensure that the program was converging. In protocol 2, the populations of the free and bound components were both varied such that there were two independent variables, the relative concentration of the two components and the value of K_{sv} for the bound component. In protocol 3, a second bound component was added, producing four independent variables, the concentrations of the two bound components and their respective values of K_{sv}. Thus, in this case, eq 2 was extended to include three components, two now associated with bound nucleotides and the third with the free nucleotide whose concentration was determined following sedimentation as described above.

A fit to the data of ϵADP bound to S1 shown in Figure 2 led to a value of 1.4 for the single independent variable K_{sv}. A potential source of error in this calculation is the experimentally determined concentration of the free, fluorescent nucleotide in solution. As an additional control, the concentration of free and bound species was also allowed to vary in eq 2 with two components (protocol 2). The least-squares fit produced a value of 0.12 for the fraction of free nucleotide and a value of 1.6 for the K_{sv} of the bound fraction. Thus the additional fit gave a concentration of the free nucleotide close to that obtained from centrifugation, and the value of K_{sv} was not markedly changed from that obtained using protocol 1. This provides additional confidence in our results. The use of the third protocol, in which there was an additional bound component, did not provide a significant decrease in χ^2, indicating that the inclusion of a second bound component was not justified. Thus, these data are adequately fit by two components, a free nucleotide that is easily quenched and a bound nucleotide which is largely protected. The important observation is that the value of K_{sv} for the bound component is greatly decreased from the value observed for the free nucleotide, indicating a very high degree of protection afforded by the surrounding protein structure. In the presence of creatine phosphate (CP) and creatine kinase (CK), the

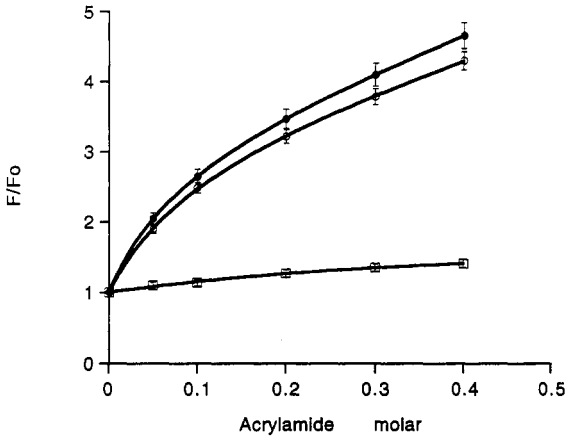

FIGURE 3: F/F_0 is plotted as a function of the acrylamide concentration for 25 μM ϵADP in the presence of 55 μM S1 in low ionic strength rigor solution in the absence (□) and presence (●) of 1 M KCl and in high ionic strength solution plus 10 mM CP and 1 mg/mL CK (○), all at 20 °C. The value of the Stern–Volmer quenching constant, K_{sv}, was 1.6 for S1 at 20 °C in low ionic strength. A reasonable fit to the data for S1 in 1 M KCl required three components: one free component, whose concentration was taken as 60% of the total from sedimentation, and two bound components, one having a K_{sv} of 16 M^{-1}, representing 40% of the bound nucleotide, and the other having a K_{sv} of 1.2 M^{-1}, representing 61% of the bound nucleotide. In the presence of CP/CK, the fit also required two bound components, one with K_{sv} = 12 M^{-1}, 42% of bound nucleotides, and the other with K_{sv} = 1.0 M^{-1}, 58% of bound nucleotides.

nucleotides underwent steady-state hydrolysis, resulting in a slight increase in the value of K_{sv} (see Table 1).

Effects of Temperature, Ionic Strength, and Nucleotide State. As shown in Figure 2, inclusion of creatine kinase and creatine phosphate, to regenerate ϵATP from ϵADP, causes little change in the degree of quenching. Thus, nucleotides undergoing steady-state cycling are protected by approximately the same degree as is a bound diphosphate nucleotide. Similar results were also found by Rosenfeld and Taylor (1984). The accessibility of the bound nucleotide to acrylamide was increased by raising the temperature or by raising the ionic strength. There is a modest increase in K_{sv} of the bound component as the temperature is raised, from 1.55 M^{-1} at 10 °C to 1.95 M^{-1} at 30 °C, indicating that the protein structure in the vicinity of the nucleotide provides less protection at higher temperatures. The change in protection is almost the same for ϵADP and for ϵATP during steady-state hydrolysis, with K_{sv} rising to 2.2 M^{-1} at 30 °C in the presence of CP and CK. The data at the higher temperature could still be fit with a single bound component using eq 2 and protocol 2.

As shown in Figure 3, data taken in the presence of 1 M KCl display a distinct curvature. An adequate fit to the data required the inclusion of two bound components, using protocol 3. The concentration of free probe, determined for the sample by centrifugation through the membrane, was a little greater than that for S1 at the lower ionic strength. The binding constant had decreased from 9×10^4 M^{-1} at the low ionic strength to 1×10^4 M^{-1} at the high ionic strength. This result is similar to that found by Konrad and Goody (1982), who also measured the effect of ionic strength on the binding of ϵADP. However, the increase in the concentration of the free nucleotide accounts for only a portion of the increased quenching seen at the high ionic strength, and a significant portion is due to increased quenching of a species of bound nucleotide. A fit to the data using protocol 3 suggests that at the higher salt concentration the S1 nucleotide complex can exist in two forms: one with a decree of protection similar to that seen at the lower ionic strength and one with a nucleotide that is far more accessible to acrylamide, suggesting that the nucleotide pocket has opened; see Table 1. The greater accessibility seen at the higher ionic strength indicates that electrostatic interactions are probably involved in the closing of the pocket. In the presence of CP/CK, the nucleotides bound to S1 with approximately the same affinity and solvent accessibility, with a major component, 58% ± 10% of bound nucleotides with $K_{sv} = 1.0 \pm 0.3$ M^{-1}, and a minor component, 42% ± 10% of bound nucleotides with $K_{sv} = 12 \pm 4$ M^{-1}.

Quenching of ϵADP Bound to Acto-S1. The measurement of the fluorescence of ϵADP bound to acto-S1 presents technical difficulties. The binding constant is decreased by almost a factor of 10, requiring high concentrations of both S1 and actin. These samples are turbid, necessitating front-face fluorescence to avoid significant artifacts due to absorption of light. Another problem, however, was encountered with these samples. It was found that the addition of ATP to displace bound ϵADP from S1 produced a change in the fluorescence intensity, which was not as great as that expected for displacement of all of the bound fluorescent nucleotide. It was determined that the fluorescent nucleotide was becoming incorporated into actin. This process was aggravated by the fact that actin had been dialyzed prior to use to remove external nucleotides, possibly removing some of the nucleotide normally bound to the actin. The presence of a fluorescent nucleotide bound to the actin complicated interpretation of the data. This nucleotide has a low value of K_{sv} because it is enclosed within the actin structure (Root & Reisler, 1992). Inclusion of phalloidin with the actin improved the ability of ATP to displace bound fluorescent nucleotides at the end of the experiment. The fraction of nucleotide bound to actin was determined following addition of ATP to displace the ϵADP from the myosin active site, and its fluorescence was subtracted from the total. The residual fluorescence was analyzed as described above, leading to a low value for K_{sv}, 1.2–2.5 M^{-1}. However, attempting to fit data that was composed of three components, some of which were changing with time, introduced significant errors. Better data for the complex with actin were obtained by observing the fluorescence of nucleotides bound to myofibrils or bound to myofibrils which had been in addition loaded with S1. In this case, there was little nonspecific binding of the fluorescent nucleotide to sites not displaceable by ADP.

The quenching of fluorescence of nucleotides bound to myofibrils is shown in Figure 4. The Stern–Volmer plot, shown in Figure 6, displays a curve with downward concavity, again signifying the presence of multiple components with different values of K_{sv}. The binding constants of ϵADP to myofibrils and to myofibrils loaded with S1 were obtained by centrifugation as described in Materials and Methods. The data listed in Table 1 define a value for the affinity constant K_a for the binding of ϵADP to myofibrils of 2×10^4 M^{-1}. The affinity increased slightly as the temperature was decreased. Upon binding of S1 to myofibrils, the value of K_a calculated from the bound component decreased to 8×10^3 M^{-1}.

As was the case with S1 described above, the data could be fit with protocol 1 in which the concentration of the free nucleotide was determined following the sedimentation of the myofibrils. Use of this protocol produced a K_{sv} for the bound fraction of 1.2 ± 0.1 M^{-1}, very similar to that obtained for S1 alone. Again, the fit using protocol 2, in which the concentrations of bound and free nucleotides are both independent parameters, produced a similar fit to the data, and inclusion of a second bound component did not produce a significantly better fit. Together, these data suggest that

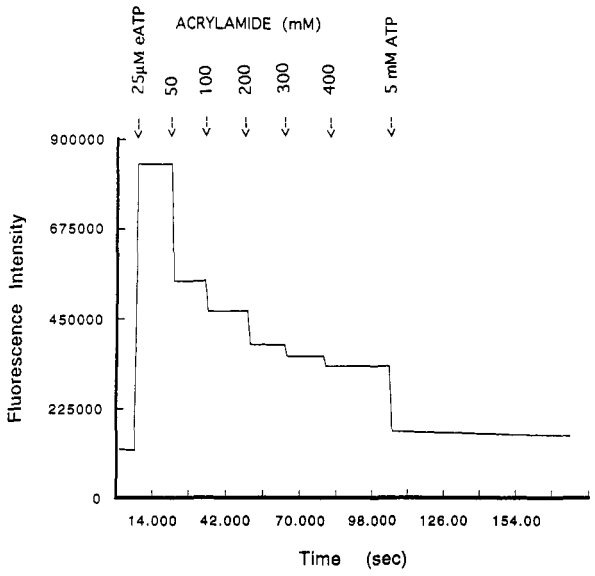

FIGURE 4: Fluorescence is shown as a function of time for 25 μM ϵADP in the presence of 20 mg/mL myofibrils. The initial signal, which comes from the proteins alone, is higher than that shown in Figure 1, due to the greater turbidity of the sample. The addition of ϵADP causes a large increase in intensity. Acrylamide was added to the concentrations shown. The decrease in the fluorescence is greater at lower concentrations of acrylamide due to the quenching of free ϵADP. At higher concentrations of acrylamide the decrease is less marked, indicating that the bound nucleotides are protected from the solvent-phase quencher acrylamide. ATP is added at the end of the run to displace bound ϵADP from active sites, providing a measure of the fraction of the signal that is due to nucleotides bound to active sites. The myofibrils were in low ionic strength rigor solution at 20 °C.

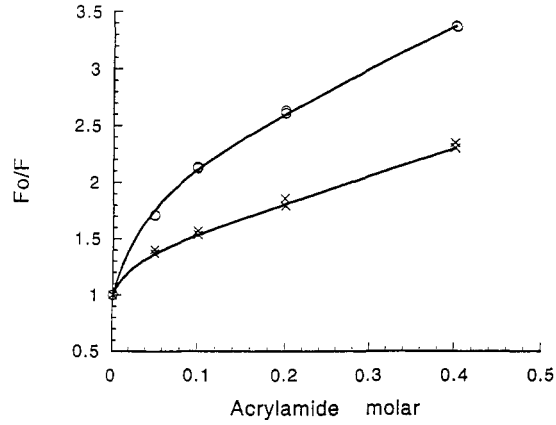

FIGURE 5: F_0/F is shown as a function of the acrylamide concentration for ϵADP bound to myofibrils (O) and bound to myofibrils which had been loaded with S1 (×). The concentration of myofibrils was 20 mg/mL, providing a concentration of myosin sites of 45 μM. The addition of S1 to the myofibrils provides an additional 70 μM S1 sites, to provide a total of 110 μM active sites. The myofibrils were in low ionic strength rigor solution at 20 °C.

the nucleotide bound to myofibrils is enclosed within the myosin nucleotide pocket, such that it is protected from collisional quenching by acrylamide. Inclusion of S1 bound to actin in the myofibrils produced a greater fraction of bound nucleotide, due to the higher concentration of myosin active sites; however, the protection provided to the bound nucleotide fraction was approximately the same as for the myofibrils alone, as shown in Figure 5 and Table 1. Thus, the data show that the binding of S1 to actin has not increased the accessibility of the fluorescent nucleotide to quenching by acrylamide. A similar result was obtained using a photoaffinity analog of ϵADP that was attached covalently to Ser-324 adjacent to the active site of myosin (Luo et al., 1994). In this case the effective

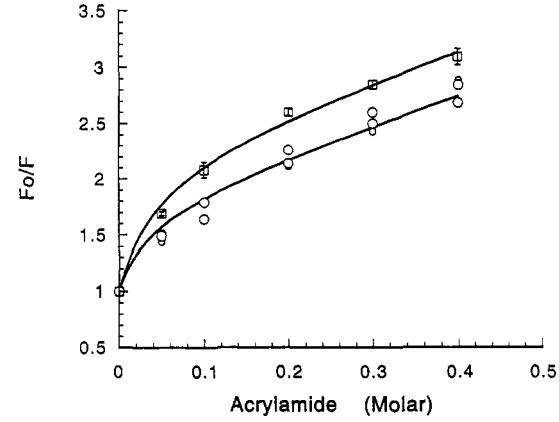

FIGURE 6: F_0/F is plotted as a function of acrylamide concentration for myofibrils in the presence of 25 μM ϵADP (□) and for myofibrils in the presence of 25 μM ϵATP undergoing steady state cycling replenished by CK and CP (O). Data are shown for an average ± SEM of six different experiments in the presence of ϵADP and for three separate experiments in the presence of CP/CK. The solution contained a low ionic strength rigor buffer, 20 mg/mL myofibrils, and either 25 μM ϵADP or ϵATP plus 1 mg/mL CK, 25 mM CP, and 1.1 mM $CaCl_2$ at 20 °C. Observation of the myofibrils in a phase-contrast microscope following the measurement of fluorescence showed that the sarcomere pattern remains intact.

concentration of the nucleotide was very high, such that binding of actin to the modified S1 caused no decrease in the proportion of nucleotide within the active site and also caused no change in the accessibility of the etheno ring to acrylamide.

Solvent Accessibility of Ethenonucleotides in Active Myofibrils. Figure 6 shows the Stern–Volmer plot obtained for active myofibrils. Addition of creatine kinase (CK) and creatine phosphate (CP) to the myofibrils regenerates ϵATP and induces active cycling. Shown for comparison are the data for the same preparation in the absence of the regeneration system. In the presence of CP/CK, the data shift to lower values of F_0/F, which can be accounted for almost entirely by the tighter binding of the fluorescent nucleotide in the active myofibrils. The value of K_{sv} is only slightly larger in the active myofibrils, as shown in Table 1.

Observation of the myofibrils in a phase-contrast microscope following the observation of fluorescence showed that the sarcomere pattern remains intact and that the myofibrils have not shortened. The lack of shortening is due to the low concentration of ϵATP, approximately 1 bound nucleotide/20 myosin active sites, and to the lower tension produced by each ethenonucleotide, about $^1/_3$ that produced by ATP (Pate et al., 1993). The active heads are unable to cause significant shortening of the myofibrils during the time course of the experiment. Thus in the preparation, myosin heads that bind nucleotide are undergoing steady-state cycling under isomeric conditions. Although many of the heads are in rigor, these heads are not observed.

The added CP was not exhausted during the experiment. A similar nucleotide, 2-aza-ϵATP, is turned over with a rate of 0.3 s^{-1} in active isometric fibers at saturating concentrations of nucleotide and at 10 °C (Pate et al., 1993). In the present experiments approximately 10 μM myosin heads had bound ethenonucleotides at any given time. Assuming that the above rate applies to ϵATP and that the rate may increase by a factor of 2–3 at 20 °C, only 5–10 μM ϵATP will be hydrolyzed per second, requiring approximately 2500 s for hydrolysis of the 25 mM CP used. The experiment typically required 150–250 s to complete.

Effects of Acrylamide and of the Etheno Ring on the Actomyosin Interaction. In order to draw conclusions from the data on active myofibrils, we must first address questions

concerning the perturbations produced on the actomyosin interaction by the use of acrylamide and a nucleotide analog. Acrylamide, even at high concentrations, appears to exert a negligible perturbation on the interaction of actin and myosin. As shown by Ando and co-workers, acrylamide concentrations up to 100 mM had no effect on either the maximum enzymatic activity or the K_m of the S1 MgϵATPase activity (Ando & Duke, 1982; Ando et al., 1982). Rosenfeld and Taylor (1984) showed that acrylamide up to a concentration of 200 mM does not affect the rate of hydrolysis of ϵADP by either S1 or acto-S1. To further explore the properties of acrylamide, we measured the effects on tension and velocity produced by addition of 400 mM acrylamide to active skinned muscle fibers. Mechanical measurements were made as described in Pate et al. (1993). Addition of 400 mM acrylamide to a skinned rabbit psoas fiber activated at 10 °C decreased tension by only 4% and decreased the maximum contraction velocity by 6%. Thus we conclude that inclusion of high concentrations of acrylamide produced a minimal effect on the actomyosin interaction.

A second question concerns how well the ethenonucleotides resemble the native substrate. Yanagida (1981) found that ϵATP produced high isometric tension in glycerinated muscle fibers. Pate et al. (1993) found that a related nucleotide, 2-aza-ϵATP, produced somewhat less isometric tension, approximately $^1/_3$ that of ATP. A similar result was found for ϵATP (Pate, White and Cooke, unpublished results). White et al. (1993) studied the kinetics of the binding and hydrolysis of 2-aza-ϵATP by myosin and acto-S1 in some detail. In general, the rates were similar to those found for ATP; however, the association rate for the binding of 2-aza-ϵATP to myosin or to actomyosin was slower than that of the native nucleotide. Together these data indicate that the ethenonucleotides function as effective substrates, but with kinetic rates, mechanics, etc. that are reduced to $^1/_3 - {}^1/_2$ those of the physiological substrate.

DISCUSSION

The present study was motivated by the structure of the myosin molecule determined by Rayment et al. (1993b). In this structure the pocket that binds nucleotides is a broad depression in the protein surface, and analogy with other proteins suggests that it should close around bound nucleotides. We sought to answer two questions concerning the conformation of this pocket. What are the energetics associated with the changes in the conformation of the pocket? Is the pocket open at any point in the active cycle, and in particular is it open when myosin is bound tightly to actin at the end of the powerstroke?

Solvent Accessibility as a Probe of the Conformation of the Nucleotide Pocket. The conformation of the nucleotide pocket was monitored via the solvent accessibility of bound fluorescent nucleotides. We used ethenonucleotides because they bind effectively, they are reasonable substrates, and they have good fluorescent properties. Acrylamide is a small uncharged molecule, MW = 71, that is an effective quencher of the fluorescence of the ethenonucleotides and produces minimal perturbation on the system. In the crystal structure of myosin the breadth of the pocket, 15 Å, is too great to provide a high degree of protection to bound nucleotides (Rayment et al., 1993a). A nucleotide that was bound to one side of the pocket in the crystal structure configuration would still be sufficiently accessible to the solvent to allow collisions with acrylamide to occur. Thus a high degree of protection would imply that the pocket had altered its conformation to more effectively enclose the nucleotide.

Is the availability of the ethenonucleotides to acrylamide proportional to the solvent accessibility of the nucleotide? In at least one case these two appear to be related. The accessibility to acrylamide of ϵADP bound to actin is approximately equal to the solvent-accessible area of ATP determined from the crystal structure (Root & Reisler, 1992). Additional support for this conclusion comes from studies of the quenching of tryptophan fluorescence by acrylamide. The degree of solvent accessibility was found to correlate with the polarity of the environment of the tryptophan, which provides an independent measurement of solvent accessibility for this fluorophore (Eftink & Ghiron, 1976). Together, the features discussed above suggest that quenching by acrylamide provides a reasonable method of monitoring the conformation of the protein structure in the vicinity of a bound ethenonucleotide.

Solvent Accessibility of Nucleotides Bound to Myosin. These studies follow earlier work which showed that when ϵADP or ϵATP bound to myosin, they were protected from quenching agents in the solvent phase (Ando & Duke, 1982; Ando et al., 1982; Rosenfeld & Taylor; 1984; Miyata & Asai, 1981). The observation that ϵATP or ϵADP are protected from solvent-phase quenchers upon binding to myosin supports the model proposed by Rayment et al. (1993b) by demonstrating that the pocket can in fact close around the nucleotide. However, an even more stringent test of this hypothesis is to measure the energetics associated with the changes in the conformation of the pocket. For myosin alone the conformation of the pocket should be tightly coupled to the binding energy supplied by the nucleotide. If the opening of the pocket provides the free energy that drives the powerstroke, the open conformation should be energetically favored by at least 30 kJ/mol in order to provide the necessary energy.

All investigators agree that ethenonucleotides bound to S1 are highly protected. However, the exact degree of protection reported has varied. Using a more limited range of acrylamide, Ando et al. (1982) reported a K_{sv} of 6.8 M^{-1}. Rosenfeld and Taylor (1984) observed two fluorescent components for ϵADP bound to S1, a minor one comprising approximately 33% with a K_{sv} of 1.0 M^{-1} and a major one comprising 66% that was less protected with a K_{sv} of 10 M^{-1}. At low ionic strength our data are adquately fit by a single component, corresponding to the more protected component observed by Rosenfeld and Taylor. However, we have fewer data points than the previous workers, and thus it is possible that a minor component with a larger value of K_{sv} was missed. In this regard, we note that some of the Stern–Volmer plots obtained at low ionic strength could accommodate a second, more quenchable component. However, most of the data appeared to be better fit without the more quenchable component, as described in the legend to Figure 2. We do observe two components as ionic strength is raised. The K_{sv} of 16 M^{-1} found for one component of ϵADP bound to S1 at high ionic strength shows that the nucleotide pocket is in a much more open conformation, with about the same accessibility as observed for the more quenchable component by Rosenfeld and Taylor (1984) but at a lower ionic strength. Hence, although the agreement between the two studies is not complete, both studies are in agreement in suggesting that the myosin pocket can exist in two conformations: a closed one in which the nucleotides are protected from the solvent and an open one in which they are largely accessible. The more open conformation is favored by higher ionic strength. Two conformations of myosin have also been proposed by Aguirre et al. (1989) and by Shriver and Sykes (1982); however, their relationship to the ones resolved by quenching is not clear.

Although the observation of both open and closed forms of the pocket suggests that transitions between them may be involved in force generation, the energetics of this transition are not appropriate for a transition that drives the powerstroke. Whether during the steady-state cycling of ϵATP or with bound ϵADP at the active site, the ratio of the closed to open conformations is similar and is equal to about 1, as found by Rosenfeld and Taylor (1984) at low ionic strength and in the present work at higher ionic strength. Thus ϵADP, which has an affinity for myosin that is orders of magnitude less than that of ϵATP, can still close the pocket. A second observation also suggests that the binding energy of the nucleotide is not tightly coupled to the conformation of the nucleotide pocket. The affinity of both tri- and diphosphate nucleotides for myosin decreases only modestly as the ionic strength is raised. (Table 1; Konrad & Goody, 1982). However, the conformation of the pocket following binding is almost completely in the closed form at low ionic strength and is partially in an open conformation in high ionic strength. Thus the free energy of binding has not been greatly altered by a shift in the conformation of the pocket. The temperature dependence of K_{sv} again suggests that the energetics of the closing of the pocket are independent of the nucleotide bound. As the temperature is increased, the pocket assumes a more open configuration; however, the temperature dependence is only slightly different during steady-state cycling, suggesting that the enthalpy associated with this transition is approximately the same for ϵADP and for ϵATP during steady-state cycling.

Together, these observations argue that, for isolation myosin, the free energy associated with opening and closing of the pocket is not large and that it is not tightly coupled with nucleotide binding. Thus the free energy driving the powerstroke is not derived directly from a transition of a nucleotide pocket that is energetically more favorable in the open state. If the energy provided by the binding of ATP is not necessary for closing the pocket, it must be used for something else. We suggest below that it is most probably used to break the tight bond between myosin and actin.

Solvent Accessibility of Nucleotides Bound to Actomyosin. The model proposed by Rayment and et al. (1993b) predicts that the nucleotide pocket should be open at the end of the powerstroke. In the crystal structure of S1, the nucleotide pocket is in an open configuration. It was assumed that it retains this configuration when the S1 binds to actin in the rigor complex, as the shape of S1 determined in the crystal fits into electron micrographs of this complex (Rayment et al., 1993b; Schroder et al., 1993). The true end of the powerstroke is thought to be the rigor complex in which the nucleotide pocket is empty [for reviews, see Cooke (1986) and Goldman (1987)]. However, addition of ADP to a rigor state does not appear to result in a large change in the orientation of muscle cross-bridges and causes little change in fiber mechanics, suggesting that this state is also near the end of the powerstroke (Rodger & Tregear, 1974; Fajer et al., 1990; Dantzig et al., 1991). Thus one test of the proposed model is to determine whether the binding of myosin to actin opens the pocket, allowing solvent-phase quenchers to reach bound ϵADP. However, measuring the fluorescence of a bound nucleotide in the actomyosin complex is technically demanding because the affinity of the nucleotide for the complex is an order of magnitude weaker than its affinity for myosin alone. This problem was solved by using high protein concentrations, front-face fluorescence, and short path length cells. A similar approach was previously successfully employed by our laboratory to measure distances within an actomyosin complex using fluorescence energy transfer (dos Remedios & Cooke, 1984).

We found that ϵADP remains largely protected from the quencher when myosin binds to actin, with a value of K_{sv} that was even slightly less than that observed for myosin alone. As discussed above, if the pocket assumed a configuration that was as open as observed in the crystal, the nucleotide would be more accessible to the quencher; thus, the above result that the bond between myosin and actin has not favored an open conformation of the pocket. In addition, the nucleotides remained protected from acrylamide during steady-state cycling in isometric myofibrils, with a modest increase in K_{sv} to a value that was similar to that observed for S1 alone during steady-state cycling. These two results suggest that the pocket remains largely closed during steady-state cycling and that it also remains closed when the cycle is arrested in a state near the end of the powerstroke. It is still possible that the pocket may open during some intermediate states in the cycle. The data of Figure 6, for myofibrils in the presence of ATP, could also be fit with protocol 3 and a third component having a K_{sv} of 16 M^{-1} and comprising as much as 10% of the total bound nucleotide. However, the observations that the nucleotide is protected in a state that precedes the powerstroke (the predominant state of S1 during steady-state cycling) and that it remains closed when arrested at a state near the end of the powerstroke make it unlikely that it opened in some intermediate state in between. Thus, we conclude that the powerstroke is not accompanied by opening of the nucleotide pocket in a single step. As discussed below, the binding and release of nucleotides is a complex sequence, and the data do not rule out an open pocket in other states. If the pocket does not open coincidentally with the transition to the end of the powerstroke, the free energy driving the powerstroke is not derived from the conformational energy associated with the opening of the nucleotide pocket. We suggest below that the energy driving the powerstroke is derived from the formation of the actomyosin interface.

Binding of actin to myosin is known to accelerate the rate of nucleotide binding and release, resulting in a 10-fold decrease in the affinity of ADP for the active site (Biosca et al. 1986; Geeves, 1989). How is this accomplished if the pocket remains closed? Binding and release of nucleotides occur from the collision complex, in which the pocket is presumably open. Our results suggest that the collision complex is followed by a transition to a state in which the pocket has closed to protect the nucleotide from the solvent. This second state would be favored over the collision complex in both myosin and actomyosin. A rapid equilibrium between the collision complex and the second state would allow for binding or release of the nucleotides, with the population of the collision complex being 10-fold greater in actomyosin than in myosin alone. Are the states populated in myosin similar to those populated in actomyosin? Some information on this question can be provided by the observed change in fluorescence of the nucleotides when bound to the protein. We found that the fluorescence of ϵADP was quenched by 10% ± 3% when bound to S1 and by 14% ± 6% when bound to myofibrils. The value for myofibrils is not statistically different from that observed with S1 alone, providing some indication that the states are similar.

Models of Actomyosin Function. An important aspect of any model for actomyosin function involves the energetics of the various chemomechanical intermediates. Ultimately, of course, the free energy comes from the splitting of ATP. However, ATP is hydrolyzed on the myosin head prior to the powerstroke, so that the energy directly driving the powerstroke must come from conformational free energy within the protein complex. Two sources are obvious candidates. One, proposed

by Rayment et al. (1993b), is the conformational energy involved in opening and closing the nucleotide pocket. The open conformation is the low-energy conformation. The pocket is then closed by the tight binding of ATP. The subsequent opening of the pocket drives the movement of the lever arm neck region. Thus in this model, the free energy driving the powerstoke is derived from that which is released when the nucleotide pocket assumes an open conformation that has substantially lower free energy than the closed conformation. This model is not supported by the data discussed above, however. The quenching of fluorescent nucleotides suggests that the energetics of the opening and closing of the nucleotide pocket are not tightly coupled to nucleotide binding for isolated myosin. More importantly, the pocket is not open at the end of the powerstroke. Thus we conclude that the energy that directly drives the powerstroke probably comes from another source. A second possible source for the necessary energy is the formation of a tight bond between actin and myosin. This possibility has been adopted by many prior theorists (Eisenberg et al., 1980; Pate & Cooke, 1988). A large body of data shows that the bond between actin and myosin in the fiber is tight and that the free energy released upon formation of this bond is sufficient to drive the powerstroke (Highsmith et al., 1976; Pate & Cooke, 1988, Geeves, 1989). The formation of an extensive interface between these two proteins, involving many hydrophobic residues, has been identified from the structure of the actomyosin complex (Rayment et al., 1993; Schroder et al., 1993). Formation of this interface could release sufficient free energy to produce conformational changes within the myosin molecule to produce the powerstroke. These conformational changes may still involve the rotation of the neck region of myosin relative to a catalytic domain which remains rigidly attached to actin throughout the powerstroke, as proposed previously both from spectroscopic probes and from an examination of the protein structures (Cooke, 1986; Thomas, 1987; Tanner et al., 1992; Rayment et al., 1993b).

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